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Course of hatch and developmental changes in thyroid hormone concentration in blood of chicken embryo following in ovo riboflavin supplementation

Karolina Barbara TRZECIAK[1], Marcin Wojciech LIS[1,*], Andrzej SECHMAN[2], Barbara PŁYTYCZ[3], Agata RUDOLF[1], Tomasz WOJNAR[4], Jerzy Wojciech NIEDZIÓŁKA[1]

[1]Department of Poultry and Fur Animals Breeding and Animal Hygiene, University of Agriculture in Krakow, Krakow, Poland
[2]Department of Animal Physiology and Endocrinology, University of Agriculture in Krakow, Krakow, Poland
[3]Department of Evolutionary Immunology, Institute of Zoology, The Jagiellonian University, Krakow, Poland
[4]Department of Animal Reproduction and Anatomy, University of Agriculture in Krakow, Krakow, Poland

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Abstract: The influence of riboflavin on the function of the hypothalamo–pituitary–thyroid axis during chicken embryogenesis is poorly understood. Therefore, examination of the effects of in ovo riboflavin supplementation on the possible linkage between the thyroid gland function and chick embryonic development seems to be interesting. Eggs on the sixth day of incubation were injected with 0 (control), 60, or 600 µg of riboflavin/egg. Blood samples were collected on the 12th, 15th, 18th, and 20th days of embryogenesis. Thyroxine (T_4) and triiodothyronine (T_3) concentrations in plasma samples were determined by radioimmunoassay method. The time of external pipping and hatching of each chick as well as the body weights of sampled and newly hatched chicks were recorded. Generally, riboflavin supplied in ovo did not affect chicken embryo mortality and hatchability; however, a dose of 600 µg of riboflavin/egg had a tendency to reduce embryo body weight. Chicks exposed to 60 µg of riboflavin/egg hatched 3.7 h earlier in comparison with controls and were characterized by a higher synchronization degree of hatching. Both applied doses of riboflavin significantly elevated T_4 concentrations in blood plasma of the chicken embryos; however, on day 20 of embryogenesis, both applied doses of riboflavin decreased T_3 levels in blood circulation. The data presented here suggest that riboflavin supplementation at the early stages of embryogenesis markedly affects embryonic development and influences thyroid hormone metabolism during the second half of embryogenesis.

Key words: Riboflavin, thyroid hormones, chick embryo

1. Introduction

Riboflavin (7,8-dimethyl-10-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl]benzo[*g*]pteridine-2,4-dione, $C_{17}H_{20}N_4O_6$, vitamin B2) plays a key role in energy metabolism as a source of flavin mononucleotide (riboflavin-5′-phosphate, FMN) and flavin adenine dinucleotide (FAD). Moreover, it is a component of the flavin enzymes and coenzymes, which carry electrons in oxidation and reduction reactions, and it also plays a key role in the respiratory chain and the oxidation of fatty acids and amino acids. Furthermore, riboflavin is involved in the Krebs cycle and the metabolism of folate, vitamin B6, (pyridoxine), and vitamin B12 (cobalamin) (1). Riboflavin plays an important role in the innate immunity of both plants and animals (1–3). The immunomodulatory role of riboflavin in avian immunity is particularly highly appreciated (3).

In vertebrates, flavin deficiencies lead to diseases such as glossitis, cheilosis, and organic acidurias (4). A deficiency of riboflavin is very rare in adult birds; however, its disappearance from the organism is associated with the overall hypovitaminosis (5–7). In avian species, riboflavin is required for proper embryonic development; therefore, it is accumulated in the developing chicken egg at an amount of about 300 µg per egg (8). Riboflavin deficiency is often related to mutations in an *Rd* gene encoding a riboflavin-binding protein (RfBP), which is responsible for deposition of riboflavin in eggs (9). In rd/rd mutants, effects of deficiency become apparent after day 10 of incubation (5). Embryo death occurs suddenly around day 13 of incubation and is the result of inhibition of activity of flavin-dependent enzymes, hypoglycemia, and impaired fatty acid oxidation. The adverse effects of the *Rd* gene mutation can be abolished by in ovo administration of free riboflavin or FMN but not RfBP (10). Riboflavin-supplemented embryos survived and developed properly, but those injected with RfBP died. This indicates that the unbound riboflavin that is injected into egg whites can be used by the developing embryo to ensure its proper

* Correspondence: rzlis@cyf-kr.edu.pl

development, while the injected apo-RfBP is detrimental. The fates of embryos were dependent on the relative amounts of the injected riboflavin and RfBP; excess of the latter diminished the availability of riboflavin to the chicken embryos, leading to their mortality (10).

In humans and other mammals, the relationship between plasma concentrations of riboflavin and thyroid hormones (THs) has been already described. It has been established that 3,3',5-triiodo-L-thyronine (T_3) regulates flavocoenzyme biosynthesis by determining the activities of flavocoenzyme-forming enzyme (11,12). Possibly, low concentrations of B vitamins adversely influence the hypophysis, pituitary gland, and/or thyroid gland functions (12).

The chicken embryo is the most useful and sensitive model for drugs, xenobiotics, and vitamin investigations (5,13–16). Nevertheless, in the available literature there are no data showing the influence of riboflavin on the function of the hypothalamo–pituitary–thyroid (HPT) axis during chicken embryogenesis. Taking into consideration that THs play a crucial role during chicken embryogenesis and affect the time of hatching and the length of incubation (17,18), the present study was designed to answer the question of whether the supplementation of the chicken embryo with riboflavin may change 3,3',5,5"-tetraiodo-L-thyronine (thyroxine; T_4) and T_3 levels in blood circulation. Consequently, in this study the effects of this vitamin on body and organ weight, as well as the hatching parameters of embryo mortality and timing of external pipping and hatching (during the last days of incubation), were determined to find out the possible linkage between the thyroid gland function (affected by riboflavin) and embryonic development.

2. Materials and methods

2.1. Experimental design

The experimental and animal procedures were approved by the Local Animal Ethics Committee in Krakow. Eggs of the parental broiler line Ross 308 (Aviagen) were used in the experiment. Before the start of incubation, eggs were numbered and weighed. Incubation was carried out in an incubator (Massales 65, Spain) under standard conditions (temperature of 37.8 °C; relative humidity of 50%), and eggs were turned once an hour at an angle of 90° during days of incubation from days 1 to 18 (E1–E18). During incubation, eggs were candled on days 6, 8, and 18 (E6, E8, and E18). During E6 candling, eggs with unfertilized and dead embryos were discarded, while in E8 candling, embryos that died after injection were removed. On E18, candled eggs with evidence of living embryos were transferred from the turning trays into hatcher baskets and incubation was continued at 37.2 °C and 55%–65% relative humidity.

The sixth day of incubation was considered optimal to carry out in ovo supplementation according to previous experiments and publications (13–15), in order to reduce the sensitivity of the embryo to manipulation and to ensure the action of administered substances for the longest period. Eggs candled on E6 with living embryos (n = 600) were divided into 3 equal groups, which were injected with riboflavin at doses of 0 (control), 60, or 600 μg/egg. The lower dose of riboflavin was based on the publication of Lee and White (10) as a minimum quantity of this vitamin sufficient for the proper development of the chick embryo, while the higher dose exceeds by about 2 times the amount of riboflavin contained in the chicken egg (8).

Riboflavin (R9504, Sigma, USA) was dissolved in 50 μL of sterile 0.7% NaCl solution (Polpharma SA, Poland). Before the injection, the shell at the site of the injection was disinfected with 70% ethanol and a window was made in the eggshell (diameter of about 5 mm). Injection was performed using a pipette with 100 μL volume tips via the air chamber at a depth of 5 mm under the chorioallantoic membrane to the albumin without injuring the blood vessels and the amnion. The riboflavin solution was stored in a light-impervious vessel. After injection, the hole was sealed with Parafilm tape (Sigma) and eggs from every group were subsequently divided to into 2 subgroups: 1) a "hatchability" subgroup for checking hatchability and course of hatch (n = 145 eggs per group) and 2) a "sampling" subgroup for tissue sampling (n = 55 per group). Incubation was then continued under normal conditions.

2.2. Hatching course and result of hatch analyses

All eggs discarded during candling or unhatched from the "hatchability" subgroups were analyzed for the stage of development and malformations. Moreover, on E18 candled eggs from these subgroups with evidence of living embryos were transferred to hatching baskets and used to monitor the course of the hatching process. The process of hatching was checked every 2 h from the 460th hour of incubation. The time of external pipping and the time of hatching of each chick were recorded. At the end of the experiment, the unhatched eggs were broken and the stage of embryonic development was noted (8,19).

2.3. Blood sampling, body and organ weight recording, and hormone analysis in plasma

Blood samples were collected from 12 randomly selected embryos of each "sampling" subgroup on E12, E15, E18 (stage before internal pipping), and E20 (stage of external pipping). Blood was sampled into test tubes with heparin (Coaparin, Polfa Warsaw Ltd., Poland). Plasma samples were kept at –20 °C until hormone determination. After blood collection, each embryo was drained and body weights were recorded. Subsequently, the heart and liver were dissected and weighed.

Thyroid hormone T_4 and T_3 concentrations in plasma samples were determined by means of radioimmunoassay using T_4 and T_3 kits (BRAHMS, Germany). The lowest limits of T_4 and T_3 assay sensitivity were 0.7 ng/mL and 0.09 ng/mL, and mean recoveries as performed in our laboratory were 96.3% and 95.0%, respectively. The intra- and interassay coefficients of variation for T_4 and T_3 analysis were 4.0% and 5.3%, and 3.5% and 6.3%, respectively. The cross-reactions of T_4 antibodies with T_3 and rT_3 (3,3',5'-triiodo-L-thyronine) were <0.2% and 5%, respectively, while with other iodothyronines and iodothyronine-like compounds they were below 0.5%. The cross-reaction of T_3 antibodies with T_4 was 0.06%, and with other iodothyronines and iodothyronine-like particles it was below 0.2%.

2.4. Statistical analyses

Results of the pipping and hatching courses were presented as medians and means ± SDs and were analyzed by the Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks for failed normality test. Data of each group were demonstrated with a linear regression of $y = a + bx$, where y is percentage of the pipped/hatched chicks; x stands for the incubation hour; a is the intercept, i.e. the estimated start of the pipping or hatching process; and b is the slope, i.e. the degree of the synchronization of pipping or hatching processes in the time (h) necessary to pip or hatch 1% of the chicks (14,15).

The hatchability and mortality data were statistically analyzed by z-test while the results of TH concentrations as well as body and organ weight were studied by 2-way ANOVA, followed by Tukey's multiple range test. The statistical analyses were performed using Sigma-Stat 2.03 (SPSS, USA) while figures were made with Grapher 8.0 (Golden Software Inc., USA). Because the radioimmunoassay revealed that there were no significant differences in plasma TH levels between male and female embryos during the incubation process, which is in agreement with previous findings (20), the data from both sexes were combined. The results were presented as mean ± SEM and were considered significant at $P \leq 0.05$ and highly significant at $P \leq 0.01$.

3. Results

3.1. Embryo hatchability and mortality

Generally, in comparison with the control group, riboflavin supplied in ovo did not affect chicken embryo mortality and hatchability, except at the dose of 60 µg/egg. In this case, many embryos died immediately after manipulation (between E6 and E8), resulting in a decrease in hatchability by 11% ($P \leq 0.05$; Table 1).

ANOVA revealed that the embryonic body weight (EBW) was significantly influenced by stage of development ($P \leq 0.01$) and riboflavin administration ($P \leq 0.05$). The first effect was evoked by a gradual increase in EBW from 9.5 ± 0.25 g on E12 to 38.4 ± 0.90 g on E20 in the control group. The second was a result of a significant reduction of EBW by the higher dose of riboflavin (600 µg/egg) by 11% and 10% ($P \leq 0.05$) on E12 and E15, respectively (Figure 1). The relationship between the EBW and stage of embryogenesis and dose of riboflavin can be described by the following equation: $y = -35.496 + 3.606x - 0.003z$, $R^2 = 0.927$, where: y is the body weight of the embryo, x is the day of incubation, and z is the dose of riboflavin.

The weight of the liver and the heart gradually increased in all groups during embryogenesis ($P \leq 0.01$), but there was no significant effect of riboflavin administration on the weight of these tissues, nor on their relative weight ($P > 0.05$; data not shown).

3.2. Course of hatching

Riboflavin treatment did not affect the time of external pipping (Table 2). However, statistical analysis of the course of hatching revealed that chicks exposed to 60 µg

Table 1. Effect of in ovo injection of riboflavin at day 6 of incubation on embryonic mortality and chicken hatchability.

Dose of riboflavin (µg per egg)		0		60		600	
		n	%	n	%	n	%
Riboflavin-treated embryos (alive at E6)		145	100.0	145	100.0	145	100.0
Embryo mortality in following periods of incubation	E6–E8	13	9.0[a]	30	20.7[b]	9	6.2[a]
	E9–E17	11	7.6[a]	13	9.0[a]	14	9.7[a]
	E18–E21	8	5.5[a]	5	3.4[a]	4	2.8[a]
Hatchability		113	77.9[a]	97	66.9[b]	118	81.4[a]

a, b: values in rows marked with different letters differ significantly ($P \leq 0.05$).

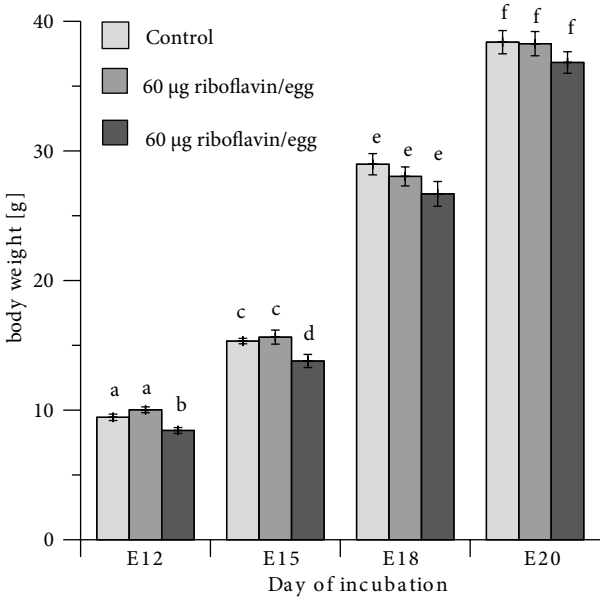

Figure 1. Body weight of chick embryos treated in ovo with riboflavin at day 6 of incubation (mean ± SE; n = 12). a, b, c, d, e, f: values in same plots marked with different letters differ significantly (P ≤ 0.05).

of riboflavin per egg hatched 3.7 h earlier than controls (P ≤ 0.05; Table 2). This was caused by a shorter length of hatching by 2.1 h (P ≤ 0.05; Table 2). Moreover, chicks from this group demonstrated a higher synchronization degree of hatching process in comparison with the controls (P ≤ 0.05; Table 2). Regression equations revealed that 1% of chicks treated with 60 µg of riboflavin needed 11.4 min for pipping and 7.2 min for hatching, while chicks of the control group needed 13.2 and 13.8 min, respectively (Table 2). Administration of riboflavin at a dose of 600 µg did not influence hatching indicators.

3.3. Hormone concentration in blood plasma

Concentration of T_4 in blood plasma of chicken embryos on E12 and E15 was relatively low; however, it significantly increased from E18 (P ≤ 0.01; Figure 2a). In the control group, on E12 it was 1.88 ± 0.13 ng/mL, and it did not change on E15 (Figure 2a). On E18, the plasma level of T_4 sharply increased, reaching a value that was 4.9-fold higher in comparison with E15 (P ≤ 0.01). An additional elevation in T_4 plasma concentration was observed on E20, as it was 1.2-fold higher in comparison to E18 (P ≤ 0.05; Figure 2a). Both applied doses of riboflavin significantly elevated T_4 concentrations in the blood plasma of the chicken embryo on E15 by about 60% (P ≤ 0.01). The stimulatory effect of riboflavin was also found on E18 and E20; however, only the higher dose of riboflavin effectively increased (by 24% and 23%, respectively) T_4 concentrations (P ≤ 0.05; Figure 2a). On the other hand, the lower dose of riboflavin (i.e. 60 µg/egg) significantly reduced T_4 levels on E12 by 16% (P ≤ 0.05; Figure 1a).

Table 2. Effect of in ovo injection of riboflavin at day 6 of incubation on the hatching process.

Stage of	Dose of riboflavin		Time of stage (hour of incubation)		Estimated start of stage (a ± S_a)	Degree of the synchronization (b± S_b)
hatching	(µg/egg)	n	median	mean ±SE	(hour of incubation)	(h)
External pipping (EP)	0	117	468^{a}	467.6 ± 0.89^{a}	456.4 ±0.50^{a}	0.22 ± 0.008^{a}
	60	99	464^{a}	466.5 ± 0.85^{a}	457.1 ±0.50^{a}	0.19 ± 0.008^{ab}
	600	120	468^{a}	469.2 ± 1.97^{a}	457.5 ±0.48^{a}	0.23 ± 0.008^{a}
Hatching (H)	0	113	476^{b}	477.1 ± 0.93^{b}	465.5 ±0.43^{b}	0.23 ± 0.020^{a}
	60	97	472^{b}	473.4 ± 0.62^{c}	467.1 ±0.54^{b}	0.12 ± 0.030^{bc}
	600	118	480^{b}	478.8 ± 0.89^{b}	467.8 ±0.49^{b}	0.22 ± 0.010^{a}
Length of hatching (DH = H – EP, h)	0	113	8^{c}	9.0 ± 0.46^{d}	-	-
	60	97	8^{c}	6.9 ± 0.66^{e}	-	-
	600	118	8^{c}	9.8 ± 0.46^{d}	-	-

Course of chicken hatching was estimated with linear regression $y = a + bx$, where y is percentage of the pipped/hatched chicks; x stands for the incubation hour; a is the intercept, i.e. the estimated start of pipping or hatching process; b is the slope, i.e. the degree of the synchronization of pipping or hatching processes in the time (h) necessary to pip or hatch 1% of chicks; S_a is the error of intercept; and S_b is the error of slope.

a, b, c, d: values in columns marked with different letters differ significantly (P ≤ 0.05).

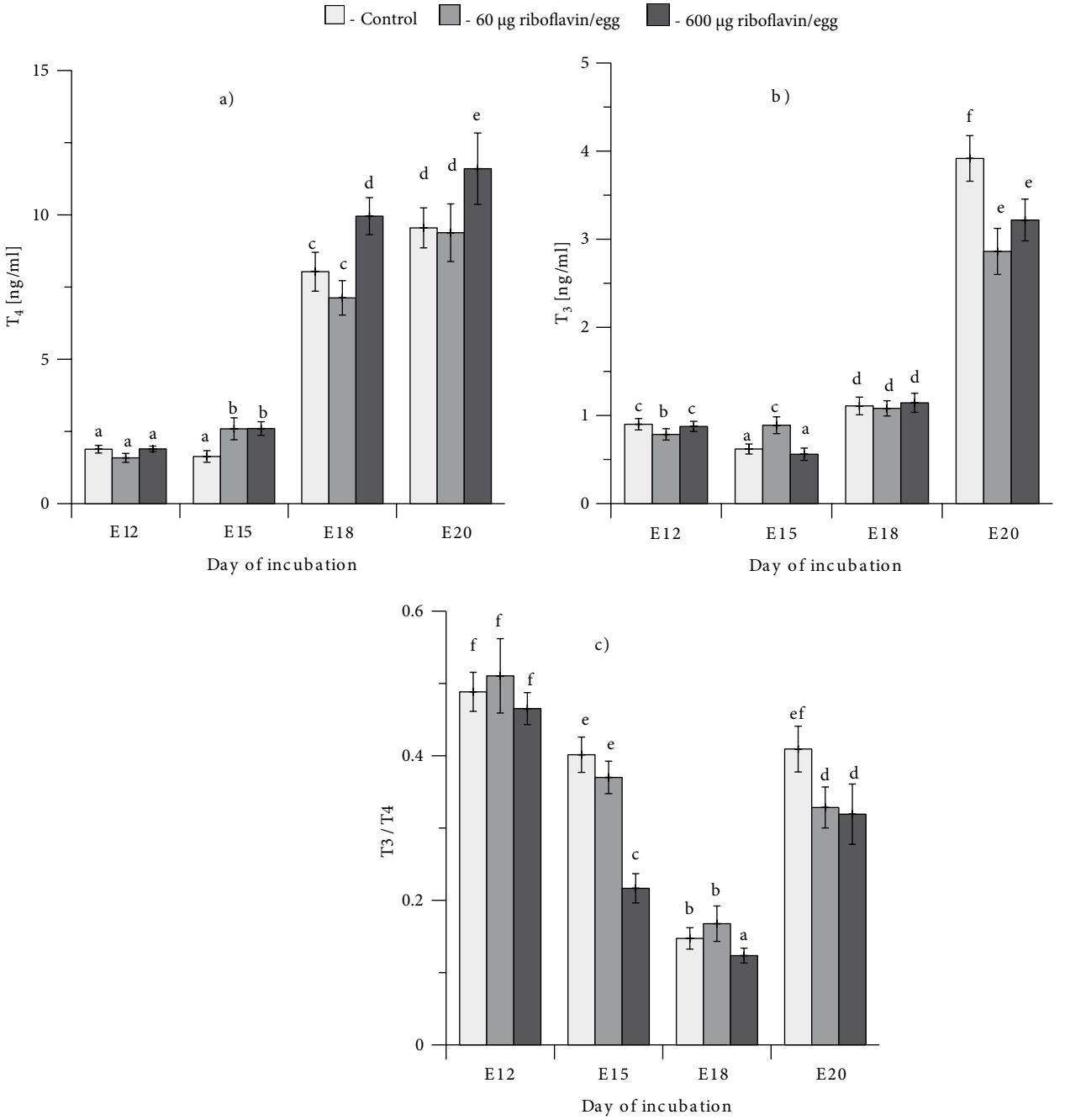

Figure 2. Concentration of thyroxine (T_4) (a), concentration of triiodothyronine (T_3) (b), and the molar ratio of T_3 to T_4 (c) in blood plasma of chicken embryos treated in ovo with riboflavin at day 6 of incubation (mean ± SE; n = 12). a, b, c, d, e, f: values in same plots marked with different letters differ significantly ($P \leq 0.05$).

In the control group, the plasma concentration of T_3 was 0.90 ± 0.06 ng/m: on E12 (Figure 2b). It was significantly decreased (1.45-fold) on E15 ($P \leq 0.05$), and subsequently, on E18, it rose by 1.8-fold in comparison to E15 ($P \leq 0.01$). A sharp increase (2.9-fold in comparison with E18; $P \leq 0.01$) in T_3 concentration in blood plasma of chicken embryos was observed on E20 (Figure 2b). A lower dose of riboflavin (i.e. 60 µg/egg) reduced the T_3 level by 13% ($P \leq 0.05$) on E12; on the other hand, it elevated concentration of this hormone by 44% on E15 ($P \leq 0.01$; Figure 2b). There were no significant alterations in T_3 levels on E18; however, on E20 both applied doses of riboflavin decreased T_3 levels in blood circulation by 27% ($P \leq 0.01$) and 19% ($P \leq 0.05$), respectively (Figure 2b).

The molar T_3/T_4 ratio is presented in Figure 2c. In the control group, a gradual decrease in T_3/T_4 ratio from 0.49 ± 0.03 on E12 to 0.15 ± 0.02 on E18 was observed ($P \leq 0.01$). A sharp 2.8-fold increase in T_3/T_4 was found on E20 ($P \leq 0.01$). The higher dose of riboflavin significantly reduced the T_3/T_4 ratio on E15, E18, and E20 by 46% ($P \leq 0.01$), 16% ($P \leq 0.05$), and 20% ($P \leq 0.05$), respectively. On E20 the lower dose of riboflavin also decreased the molar ratio of T_3/T_4 by 19% ($P \leq 0.05$; Figure 2c).

4. Discussion

The results of this experiment reveal that the injection of riboflavin at the beginning of embryogenesis may disturb the course of chick embryo development. It was found that only the lower dose of riboflavin (i.e. 60 µg) decreased hatchability by 11%, which was evoked by a significant elevation in embryonic mortality (by 20.7%) from 1–2 days following the manipulation. Taken together with the fact that the mortality among the 3 groups did not differ on the following days, this suggests that the observed effect could also be due to an uncontrolled external factor rather than embryo toxicity. This explanation can be supported by fact that the sensitivity of the chicken embryo to in ovo manipulation is very high at early stages of embryogenesis and it decreases gradually during embryonic development. It is thought that disturbance in embryo homeostasis caused by the applied in ovo manipulation is the main reason for its mortality (13). Nevertheless, results of experiments performed by Lee and White (10) indicate that the fate of an embryo is dependent on the proper ratio of RfBP and free riboflavin. In ovo manipulation might disturb this ratio, and the temporal excess of apo-RfBP might be detrimental to embryos. We may speculate that this is one of the reasons for mortality of embryos injected with saline or riboflavin during the present experiments. Perhaps the low dose of exogenous riboflavin predisposes RfBP to diminish its binding properties.

Several lines of evidence indicate that a reduction in body weight during the pre- and postembryonic period and in chickens (6,21) is associated with a riboflavin deficiency. In sharp contrast, in our experiment the highest dose of riboflavin (i.e. 600 µg/egg) substantially decreased the weight of the chick embryo. A reduction in gains was also observed in the growing broiler chicken supplemented in ovo with 1.5 and 3 mg of riboflavin (22). It cannot be excluded that the reduction in body weight following riboflavin treatment is associated with an increase in the basal metabolic rate as a result of elevation in the activity of flavin-dependent enzymes (i.e. flavoprotein monooxygenases, acyl-CoA dehydrogenases, or cytochrome P450 reductase) (1). Moreover, riboflavin is a cofactor in the conversion of pyridoxine (vitamin B6) to pyridoxal phosphate. It seems to be likely that an excess of riboflavin accelerates depletion of available pyridoxine. Deficiency of vitamin B6 can also be a cause of chicken BW loss (21).

The statistical analysis of the course of pipping and hatching reveals that the elevation in riboflavin availability on E6 may affect the rate of hatching. It can be assumed that this is related to changes in the embryonic metabolism associated with alterations in TH concentration in blood circulation. It is well known that THs play an important role in the development of many systems in all vertebrates, including birds (23,24). The rapid increase in T_3 at the end of incubation is necessary not only for stimulation of growth and differentiation, but also for preparation of the chick for a life outside the egg by regulating processes such as yolk sac retraction, the onset of pulmonary respiration, hatching, and the initiation of endothermic responses (25,26). The low T_3 concentration observed during most of embryogenesis is a result of high activity of D3 deiodinase, which in the liver and kidney converts T_3 to 3,3'-diiodo-L-thyronine (3,3'-T_2). A sharp increase in T_3 concentration, which appears slightly later in comparison with the peak of T_4, occurring during the hatching period, is associated with the transition of the chick embryo from chorioallantoic to pulmonary respiration. It is associated with a decrease in activity of D3 deiodinase and elevation in activity of D1 deiodinase, which metabolizes T_4 to T_3 (23,24).

In the present experiment alterations in TH concentration correspond with the changes described above; however, it should be noted that the release of T_4 from the thyroid gland into the blood circulation during hatching (i.e. on E20) in the group treated with riboflavin at a dose of 600 µg/egg was significantly higher in comparison with the control group. On the other hand, at the moment of pipping, the concentrations of T_3 in blood plasma in both experimental groups were significantly lower than in the control group. It can be concluded that the observed changes in T_4 levels are the result of HPT axis activity under the influence of riboflavin and changes in the metabolism of this hormone in peripheral tissues such as the liver. Recently, Grommen et al. (27) revealed that the elevated plasma T_4 levels observed during the last trimester of chicken embryogenesis associated with the increased synthesis and secretion of T_4 by the thyroid gland are caused by the increase in thyroglobulin, sodium/iodine symporter, and thyroid peroxidase mRNA expression. Therefore, the increased concentration of T_4 on E18 and E20 in the group treated with the highest dose of riboflavin might be connected with a direct influence of this vitamin on expression of thyroid-specific genes. Nor can it be excluded that the increase in T_4 concentration with a concomitant decrease in T_3 level could be evoked by the elevation in the iodotyrosine deiodinase (IYD) activity as a result of increased availability of FMN as a cofactor

for this enzyme. The IYD is the only flavin-dependent deiodinase that facilitates the recovery of iodide in the thyroid tissue by catalyzing deiodination of mono- and diiodotyrosine (28,29). However, in order to verify this hypothesis, further studies are needed.

Because in avian species almost all circulating T_3 is of peripheral origin (24), it can be assumed that the changes in T_3 levels following the riboflavin administration are the result of its impact on the activity of D1 and D3 deiodinases. The decrease in T_3/T_4 ratio in the blood of embryos treated with vitamin B2 supports this assumption and suggests that riboflavin directly increases the activity of D3 deiodinase in the final stages of embryogenesis.

The existence of the specific relationship between riboflavin and thyroid gland function observed in our experiment has already been postulated. In mammals, it has been shown that there is a correlation between the levels of riboflavin and concentration of iodothyronines in the blood circulation. In humans and rats with hypothyroidism, FAD levels decrease in the liver; they are similar to those observed in animals fed a vitamin B2-deficient diet. This phenomenon results from the T_4-related activity of flavokinase, the enzyme responsible for the conversion of vitamin B2 into FMN and FAD. Although in the hyperthyroid state the activity of this enzyme is doubled, there is no increase in the level of FMN and FAD as a result of their increased expenditure (30). Therefore, hormone replacement therapy in adults with hypothyroidism normalizes the metabolism of riboflavin. However, this treatment in newborns with congenital thyroid dysfunction does not change the levels of vitamin B2 and FMN in the blood, in spite of obtaining the appropriate level of iodothyronines (30).

In summary, riboflavin administration at the early stages of embryogenesis markedly affects embryonic development and influences thyroid hormone metabolism during the second half of embryogenesis. Negative effects of riboflavin on hatching success in the present experiments seem to be related to the early timing of its administration. Beneficial effects of riboflavin on posthatch immunity may be expected in birds supplemented in ovo at the final stages of development, as has happened in the case of vitamin E (3). In order to understand the molecular mechanism of riboflavin action in the chicken embryo, more research is needed

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